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(54) Title: NOVEL COMPOSITIONS CONTAINING HYALURONIC ACID ASSOCIATES AND A PROCESS FOR PREPARING SAME

(57) Abstract

The invention relates to novel associates (complexes) of deprotonated hyaluronic acid and 3d metal ions of the 4th period of the periodic table and compositions containing these associates (complexes) as active ingredients or carriers. The invention further relates to a process for the preparation of the above novel associates (complexes) and compositions (pharmaceutical and cosmetic compositions) containing these associates (complexes) as active ingredients. In these compositions, zinc hyaluronate is preferably used as active ingredient.

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NOVEL COMPOSITIONS CONTAINING HYALURONIC ACID ASSOCIATES AND A PROCESS FOR PREPARING SAME

The invention relates to novel associates (complexes) of deprotonated hyaluronic acid with 3d metal ions of the 4th period of the periodic table and compositions containing these associates (complexes) as active ingredients.

The invention further relates to a process for the preparation of these novel associates (complexes) and compositions containing these associates (complexes) as active ingredients.

According to a particularly preferred embodiment of the process of the present invention the aqueous solutions containing the novel associates of deprotonated hyaluronic acid with 3d metal ions of the 4th period of the periodic table are directly prepared from an aqueous solution of sodium hyaluronate.

The novel associates according to the present invention mainly involve zinc and cobalt hyaluronate. The compositions containing these latter associates may be pharmaceutical (therapeutical) or cosmetic and optionally other compositions. Fields of indication of the compositions containing the novel associates according to the invention are e.g.: the acceleration of epithelization of epithelium-deficient body surfaces; healing of crural ulcer, decubitus (bed-ulcer), primarily

not healing wounds, burns, radiation- or heat-induced wounds, vulgar acne and comglobated acnes, though they can be used in other areas, too.

Hyaluronic acid is a macromolecule known for more than fifty years which had first been described by Meyer et al. /J. Biol. Chem. 107, 629 (1954); J. Biol. Chem. 114, 689 (1936)/. The structure determination was performed by Weissman et al. /J. Am. Chem. Soc. 76, 1753 (1954)/. Hyaluronic acid is a highly viscous native glucosaminoglycan containing alternating β_{1-3} glucoronic acid and β_{1-4} glucosamine moieties; its molecular weight is between 50000 and several (8 to 13) millions. The recovery of hyaluronic acid is an old task the separation and use of an extrapure hyaluronic acid are described e.g. in the United States patent specifications Nos. 4,141,973 and 4 303 676 and in the European patent specification No. 0 144 019. Up to the last years hyaluronic acid has been employed as sodium salt e.g. in the therapy, mainly in the opththalmology, surgery and cosmetics. The salts of hyaluronic acid formed with alkaline, alkaline earth, magnesium, aluminium, ammonium or substituted ammonium ions may serve as carriers for promoting the absorption of drugs (see the Belgian patent specification No. 904,547). Heavy metal salts of hyaluronic acid (wherein "heavy metals" mean the elements of the 5th, 6th and 7th periods of the periodic table as well as the lanthanides and actinides) and within these the silver salt

are utilized as fungicidal agents whereas the gold salt is employed for the treatment of arthritis (see the patent specification WO 87/05517).

It was proven by various structure-elucidating methods that the secondary structure, i.e. the conformation of hyaluronic acid is changed by binding metal ions /W. T. Winter and A. Sruther: J. Mol. Biol. 517, 761 (1977); J. K. Sheehan and E.D.T. Atkins: Int. J. Biol. Macromol. 5, 215 (1983); and N. Figueroa and B. Chakrabarti: Biopolymers 17, 2415 (1978)7. Significantly varying effects on the molecular structure can be exerted even by metal ions of similar character as shown by comparative X-ray study of potassium and sodium hyaluronate (A. K. Mitra et al.: J. Macromol., Sci. Phys. 824, 1 and 21 (1985)7. This is much the more valid for compounds of hyaluronic acid formed with metal ions of various sorts bearing various charges.

No reference relating to associates (complexes) of hyaluronic acid formed with 3d metal ions of the 4th period of the periodic table can be found in the literature; actually, according to gel filtration chromatography examinations hyaluronic acid, in opposition to heparin, is unable to bind zinc ions (R. F. Parish and W. R. Fair: Biochem J. 193, 407 - 410 (1981)7.

In spite of the fact that, according to the literature, hyaluronic acid (or its sodium salt) is unable to bind zinc ions, the authors of the present

invention undertook to investigate the coordination chemistry of the interaction between hyaluronic acid and 3d metal ions of the 4th period of the periodic table and within these chiefly zinc and cobalt ions. Since hyaluronic acid is nearly exclusively commercialized as its sodium salt thus being the basic substance of all systems containing hyaluronate, our investigations were begun on the interaction of sodium ions and hyaluronate. For this purpose the free sodium ion activity of aqueous sodium hyaluronate solutions was measured by using a sodium selective glass electrode. It was unambiguously stated from these measurements that not more than 60% of sodium ions introduced as equivalent together with the carboxylate groups of hyaluronate are present as free ions in the aqueous solutions whereas the remainder of 40% is in the form bound to the hyaluronate.

According to our measurements, by increasing the sodium ion concentration the amount of the sodium ions bound can be raised to 50 - 55 % calculated for all available carboxylate groups. Thus, it has been verified that, differently from the common properties of salts, sodium hyaluronate is not completely dissociated in aqueous solution.

In the next step of our investigations an aqueous solution of sodium hyaluronate was titrated with zinc chloride solution by using a sodium ion-selective electrode mentioned above for following

the change in the activity of free sodium ions in the system. A characteristic curve reflecting the process is shown in Figure 1. It is perceivable that sodium ions originally bound to hyaluronate are liberated on the effect of zinc ions. Based on the results of these measurements the total sodium ion concentration is liberated by an equivalent amount of zinc, a fact unequivocally proving that zinc ions are more strongly bound to hyaluronate than are sodium ions. Thus, the earlier statement that hyaluronic acid would be unable to bind zinc ions /R. F. Parrish and W. R. Fair: Biochem. J. 193, 407 (1981)/ has experimentally been refuted.

Thereby, a knowledge of men skilled in the art was disproved, which has been valid up to the present.

From our investigations discussed above it became clear that, through the interaction of equivalent amounts of sodium hyaluronate and zinc ions (zinc chloride) in aqueous solution a zinc hyaluronate associate with a stoichiometric composition is formed. After an appropriate isotonization the solution obtained can directly be used for therapeutical purposes and the zinc compound has not to be prepared in solid state in a separate process. Preliminary examinations carried out by using cobalt ion and other 3d metal ions led to similar results.

Nevertheless, the complex was prepared in solid state for characterization and the direct environment of the zinc ion was determined by using the 'Extended X-ray Absorption Fine Structure" (EXAFS) method. It has been stated that zinc is surrounded by four oxygen atoms in the first coordination sphere.The length of the Zn-O bond distances is 199 pm whereas two carbon atoms are present in a longer distance of 241 pm from the zinc atom.

According to our examinations zinc hyaluronate significently differs from the analogous copper complex which latter contains four equatorial and two axial Cu-O bonds with the values of 194 and 234 pm, respectively. The distance between the copper atom and the next two carbon atoms is 258 pm. The structure of the cobalt complex is similar to the zinc complex but not to the copper complex.

Thus, the present invention relates to associates (complexes) of deprotonated hyaluronic acid with 3d metal ions of the 4th period of the periodic table.

The invention further relates to a composition containing as active ingredient or carrier an associate (complex) of hyaluronic acid with 3d metal ions of the 4th period of the periodic table, optionally in admixture with other active ingredient(s) and/or additives.

According to an other aspect of the invention there is provided a process for the preparation of the novel associates (complexes) of the invention, which comprises

a) adding an aqueous solution containing the equivalent amount of a salt, preferably the chloride of one of 3d metal ions of the 4th period of the periodic table to an aqueous solution of sodium hyaluronate or to an other salt (alkaline or alkaline earth metal salt, optionally silver salt) of hyaluronate; or

b) dissolving an associate formed from hyaluronic acid with a quaternary ammonium salt in an aqueous suspension in a solvent couple containing the aqueous solution of a 3d metal ion of the 4th period of the periodic table and a solvent which is partially miscible with water, preferably n-butanol; then

- precipitating the associate (complex) obtained of hyaluronic acid with the 3d metal ion of the 4th period of the periodic table by an alkanol or alkanone in a known manner, or
- separating the precipitate from the solution and then, if desired
- drying it under mild conditions.

Based on those said above, a process has

been developed for the preparation of aqueous solutions containing as active ingredient a zinc hyaluronate associate (complex) or a similar associate of a 3d metal ion of the 4th period of the periodic table, respectively. These solutions were in each case prepared by the direct reaction of the metal ion with the hyaluronate component. This method of preparation made unnecessary to previously separate the active ingredients mentioned above in a solid state. In the solution prepared by using the process of the invention the amount of free (metal-unbound) hyaluronate is negligible even in the presence of an equivalent amount of zinc. On effect of an excess of zinc ions the formation of the zinc hyaluronate associate (complex) becomes quantitative.

In the course of preparation of the metal associates in the way discussed above the pH remains at a value of about 5. In the case of a 0.2 % by weight/volume (wt./vol.) hyaluronate solution the pH reaches a value of 5.4 whereas in the case of 0.5 % by wt./vol. the pH value is 5. When necessary, the pH of the latter system can be adjusted to a value of 5.5 to 5.6 by adding a few drops of an isotonic sodium acetate solution.

Solutions of two sorts containing zinc hyaluronate as active ingredient have been prepared by using the process discussed above.

1. Zinc hyaluronate solution made isotonic by an excess of zinc chloride:

Taking into consideration that free zinc chloride alone may also preferably be used in the dermatology, the osmotic pressure of the zinc hyaluronate solution was adjusted to the isotonic value by using an excess of zinc chloride. The solution thus obtained did not contain any free (zinc-unbound) hyaluronate at all but an excess of zinc chloride was present in the system together with zinc hyaluronate.

2. Zinc hyaluronate solution made isotonic by a monosaccharide or a sugar alcohol:

For a therapeutic use wherein the presence of hyaluronate-unbound zinc ions is not indicated, the solution containing zinc ions in an amount equivalent to the hyaluronate was made isotonic by using a polyalcohol (sugar alcohol, preferably sorbitol) or a mono- or disaccharide (preferably glucose). The free zinc ion and free hyaluronate content of these latter systems did not reach 5 % of the total zinc or total hyaluronate content, respectively.

In the course of utilizing the associates according to the invention ion-free compositions may eventually be required. Namely, the associates prepared according to the above process of the invention usually contain sodium chloride or an other salt formed from the starting hyaluronate cation and the anion of the 3 d metal salt.

Two different process variants can be used for the preparation of a salt-free hyaluronic acid associate formed with a 3d metal ion. These are as follows.

a) A solution of a quaternary ammonium salt is portionwise added to the solution of a known hyaluronate, preferably sodium hyaluronate. After a satisfying purification, the novel quaternary ammonium hyaluronate associate precipitated is dissolved under vigorous stirring in a solvent couple consisting of an aqueous solution of a 3d metal ion of the 4th period of the periodic table and a solvent which is partially miscible with water, preferably n-butanol. The two phases are allowed to separate, then the hyaluronate associate is precipitated by adding an alkanol or alkanone to the aqueous phase, the precipitate is separated and washed; or

b) after adding 2.0 to 3 volumes of a C_{1-3}alkanol or C_{3-4}alkanone under stirring to a zinc hyaluronate solution, suitably to a not isotonized solution containing zinc chloride in an amount equivalent to the hyaluronate, the zinc hyaluronate precipitated is filtered and washed with the alkanol or alkanone, respectively used for the precipitation. When necessary, the

zinc hyaluronate is dissolved in ion-free water and the precipitation is repeated.

When a solid ion-free zinc hyaluronate is needed, the precipitate is dried under reduced pressure under mild conditions. In the case of a demand on an ion-free zinc hyaluronate solution it is preferable to dissolve the zinc hyaluronate made free from the solvent. According to any of both process variants an ion-free solid or dissolved product is obtained with an optional purity depending on the quality of the starting zinc hyaluronate.

The results of the clinical-pharmacological investigation on a composition (Example 13) containing as active ingredient the zinc hyaluronate according to our invention are shown on a crural ulcer treatment used for the acceleration of epithelization of epithelium--deficient surfaces. A composition containing sodium hyaluronate was used as control.

This examination was carried out on 12 or 14 ulcers, respectively of 8 or 12 patients suffering from crural ulcer. The distribution of the patients of both group accoding to sex and age as well as to the nature of the disease was as follows.

Active ingredient	No. of patients	Woman	Man	Average age	No. of ulcers treated	Character of the ulcer*		
						A	V	M
Zinc hyaluronate	12	10	2	63.9	14	2	9	1
Sodium hyaluronate	8	6	2	65.7	12	-	7	1

* A = arterial; V = venous; M = mixed

The treatments were performed in such a way that before beginning the treatment a purifying therapy was carried out according to the actual clinical state of the ulcer. The treatment with zinc or sodium hyaluronate, respectively, was commenced on ulcers nearly purified or in cases where a significant diminution of the sorbes was observed. The treatment was carried out daily once in such a way that the composition was dropped onto the surface of the purified ulcer in an amount wetting the surface of the wound with a thin layer.

The composition was used for 4 weeks. At the beginning of the treatment and then once in a week the data sheet was filled out and the ulcers of the patients were documented by photographs. A discharge sample was taken for bacteriological examination.

The characteristics as well as the severity of the epithelial lesions were marked with the following symbols and scores.

Characteristics	Severity
Area (a)	
0	0
Below 10 cm^2	1
Between 10 cm^2 and 25 cm^2	2
Above 25 cm^2	3

Characteristics	Severity
Infectedness (b)	
Clinically pure	0
Coated in 50%	1
Coated in 100%	2
Necrosis (c)	
(only in the case of an arterial ulcer)	
negative	0
Below 10%	1
Between 10% and 15%	2
100%	3
No necrosis	4

Evaluation

For evaluation the values of the separate characteristics were determined and the general severity score was calculated by using the following formula:

$$s = \sqrt{a \times b \times c}$$

The results of the clinical pharmacological investigations are illustrated in Figure 2. The results of the treatment with zinc hyaluronate is shown on the curve marked with a cross whereas that of the treatment with sodium hyaluronate is illustrated on the curve denoted with a square as a function of the number of weeks involving the treatment. The score

value plotted on the ordinate represents the general severity index calculated by using the above formula.

For a more correct comparison of zinc hyaluronate to sodium hyaluronate used as control the relative correct values related to the starting score values as 100% are illustrated in Figure 3.

The change in the relative correct values was statistically evaluated as a function of number (1 to 4) of weeks. On the zinc and sodium hyaluronate treatment, the number of ulcers decreased below a relative score value of 90%, 80%, 70% and 60%, respectively after 1, 2, 3 and 4 weeks was investigated. The results are summarized in Table 1.

Table 1

Active ingredient of the composition	1st week		2nd week		3rd week		4th week	
	Distribution of the relative score value							
	90%		80%		70%		60%	
	below	above	below	above	below	above	below	above
Zinc hyaluronate	12	2	11	3	11	3	11	3
Sodium hyaluronate	4	8	7	5	6	6	3	9

It can be stated from Table 1 that the treatment with zinc hyaluronate was in every week advantageous in comparison to the results obtained with sodium hyaluronate used as control.

The statistical analysis of the response obtained for the hypothesis in question proved that the advantage of the zinc hyaluronate composition was highly significant (p 99%) in comparison to sodium hyaluronate.

In a further statistical working-up, a more detailed destribution of the relative score values was investigated as a function of the time of treatment. The results obtained are summarized in Table 2.

Table 2

Active ingredient of the composition	Number and score value of the ulcers			
	above 90%	between 90 and 70%	between 70 and 50%	below 50%
1st week				
Zinc hyaluronate	2	7	5	0
Sodium hyaluronate	8	3	0	1
2nd week				
Zinc hyaluronate	0	6	7	1
Sodium hyaluronate	4	3	5	0

Table 2

(contd.)

Active ingredient of the composition	Number and score value of the ulcers above 90%	between 90 and 70%	between 70 and 50%	below 50%
3rd week				
Zinc hyaluronate	0	1	8	2
Sodium hyaluronate	2	5	5	1
4th week				
Zinc hyaluronate	2	1	7	3
Sodium hyaluronate	1	5	3	3

The data of Table 2 similarly support the advantage of zinc hyaluronate. The more detailed statistical examinations show the significance to decrease depending on the time of treatment.

Summing up: on an evaluation of the clinical-pharmacological investigations the higher efficiency of zinc hyaluronate could be proven even at a low number of ulcers; this advantage could particularly be supported in the starting period of the treatment.

The invention is illustrated in more detail by the following non limiting Examples.

The protein content of hyaluronate (HA) was determined by using the method of O.H. Lowry /J.

Biol. Chem. 193 (1951)7; the viscosity of hyaluronate was measured in an Ostwald's viscometer in a physiological saline solution at 25 °C. The value of the intrinsic viscosity extrapolated to "0" concentration, i.e. $[\eta]_{25}^{c\to 0\%}$ is given below. The HA content was determined by using Bitter's method [Anal. Biochem 4, 330 (1962)].

Example 1

Preparation of a zinc hyaluronate solution

40.18 mg of sodium hyaluronate are dissolved in 20.0 ml of twice distilled water. Thus, the starting concentration of hyaluronic acid is 2.009 mg/ml, the equivalent concentration of the solution is 4.241×10^{-3} mol/litre (Na^+ or hyaluronic acid dimer unit). In the course of the measurement, a zinc chloride solution of 0.05154 mol/litre concentration is added to the reaction mixture through a microburet. The solution is first added in little portions (0.05 ml) and then in larger portions(0.1 to 0.2 ml). The potential change in the solution is measured by using a precision potentiometer with digital display and sodium ion-selective glass and silver/silver chloride electrodes. The titration is continued until the potential measured is not further changed by adding an additional portion of the titrating solution. (The measuring system was calibrated under conditions analogous to the practical measurement.)

The selectivity of the sodium ion-selective electrode was observed also in the presence of Zn^{2+} ions in order to control that the potential change in the practical measurement was caused by the liberated Na^+ ions and not the Zn^{2+} ions introduced to the solution. A 2.00×10^{-3} M sodium chloride solution was titrated by using the zinc chloride titrating solution under conditions similar to the above conditions. On increasing the concentration of Zn^{2+} from 0 up to 4×10^{-3} mol/litre a potential increase of about 2 mV was observed whereas the practical measurement showed a change of about 20 mV under similar conditions. Thus, the evaluation had no obstacle. In the course of measurement the increase in the sodium ion activity calculated from the measurement data verified the quantitative formation of the zinc associate.

Preparation of a zinc chloride solution

Since a solution containing zinc chloride in an accurate concentration cannot be prepared by direct weighing-in, first a solution with the nearly desired concentration is prepared. On preparing this solution no acid should be used thus it may occur that the zinc chloride weighed in will not completely be dissolved. After sedimentation of the insoluble residue (about 30 minutes) the volumetric flask is filled up to the mark and the solution is filtered through a filter paper.

The accurate concentration of the filtrate is determined by complexometric titration by using buffer 10 and eryochrom black-T indicator. The zinc chloride solution with an accurate concentration of 0.100 mol/litre is prepared by the precise dilution of this solution.

The characteristics of sodium hyaluronate used for the preparation of solution are as follows:

Molecular weight	: 1850000 daltons
Protein content	: 0.07 % by wt.
UV absorption $A^{1\%}_{257}$	: 0.133
$A^{1\%}_{280}$	: 0.075
Viscosity $[\eta]_{25^oC}^{c \to 0}$	: 13.7 dl/g
HA^x content	: 98.12 % by wt.

^xHA = hyaluronic acid (as abbreviated herein)

Example 2

Preparation of a solution for dermatologic and cosmetic use

12.5 ml of a zinc chloride solution of 0.100 mol/litre concentration prepared with ion-free water are added to 0.50 g of sodium hyaluronate weighed in a 100 ml volumetric flask. (An other concentration of zinc chloride may also be used but the amount of zinc chloride should be the same.) Sodium hyaluronate is allowed to swell (for 12 hours) in the solution filled up to the mark with ion-free water to obtain a zinc hyaluronate solution of 0.5 % by wt./vol.

The characteristics of sodium hyaluronate used for preparing the above solution are as follows:

Viscosity $[\eta]^{c \longrightarrow 0\%}_{25\ ^oC}$: 16,5 dl/g

Protein content : 0.8% by wt.

Example 3

Preparation of a zinc hyaluronate solution for use in injectable solutions

The operations described in this Example are carried out under sterile conditions.

5.0 ml of a zinc chloride solution of 0.100 mol/litre concentration prepared with twice distilled water (water for injection use, pyrogen-free, sterile) are added to 0.20 g of sodium hyaluronate (of pure powder quality) weighed in a 100 ml volumetric flask, then the volume is filled up to 50 ml with twice distilled water. Sodium hyaluronate is allowed to swell overnight, then dissolved by shaking and the solution filled up to the mark with twice distilled water. The solution obtained is filtered through a membrane filter (0.45 µ pore size) to give a zinc hyaluronate solution of 0.2% by wt./vol.

The characteristics of the sodium hyaluronate used for preparing the above solution are as follows:

Quality : pure, pyrogen-free sterile powder

Molecular weight: 1850000

Protein content : 0.07 % by wt.

UV absorption $A_{257}^{1\%}$: 0.133

$A_{280}^{1\%}$: 0.075

HA content : 98.12 % by wt.

Viscosity $[\eta]_{25}^{c\rightarrow 0\%}{}_{C}$: 13.7 dl/g

Example 4

Preparation of an ion-free zinc hyaluronate solution

600 ml of ethanol of analytical grade are added under stirring to 200 ml of 0.50 % by wt./vol. zinc hyaluronate solution obtained according to Example 2, the precipitated zinc hyaluronate is filtered on a glass filter, washed twice with 50 ml of ethanol each of the same quality and then dried under reduced pressure. Thus, 0.88 g of zinc hyaluronate is obtained which is used for preparing a 0.50 % by wt./vol. zinc hyaluronate solution in the way described in Example 2. The zinc hyaluronate solution obtained does not contain any sodium chloride arising from the reaction between sodium hyaluronate and zinc chloride; thus, it is practically ion-free.

Example 5

Preparation of ion-free zinc hyaluronate or its solution for therapeutical use

The operations described in this Example are carried out under sterile conditions.

1500 ml of ethanol (purest quality) are portionwise added to 500 ml of zinc hyaluronate solution prepared according to Example 3 under stirring. After the addition the system is stirred for 30 minutes, the zinc hyaluronate precipitate is filtered on a glass filter, washed 3 times with 100 ml of ethanol (purest quality) each and dried under reduced pressure under mild and sterile conditions.

Example 6

Preparation of ion-free zinc hyaluronate

200 ml of 10% by wt. solution of Hyamine^{R} 1622 (puriss) (benzyldimethyl/-2-/2-p-(1,1,3,3--tetramethylbutyl)phenoxy/ethoxy/ethyl/ammonium chloride) are added under stirring to the solution containing 1 g of sodium hyaluronate in 400 ml of twice distilled water. The precipitate i.e. the hyaluronic acid quaternary ammonium associate formed is separated by centrifuging, washed twice with 100 ml of twice distilled water each and again centrifuged. The washed precipitate is dissolved in a solvent couple consisting of 400 ml of 2 % by wt./vol. zinc chloride in aqueous solution(pH 5.0 to 5.4) and 400 ml of n-butanol. After allowing to separate the two phases, the aqueous layer containing the dissolved zinc hyaluronate is filtered through a membrane filter (0.45 µ pore size), then zinc hyaluronate is precipitated by adding 3 volumes of ethanol, filtered on a glass filter, washed with ethanol and

dried in a nitrogen atmosphere under mild conditions to obtain 0.82 g of zinc hyaluronate.

When necessary, a 0.50 % by wt./vol. solution is prepared from the zinc hyaluronate obtained which is then further purified as described in Example 4. The characteristics of sodium hyaluronate used as starting material are as follows:

Viscosity $[\bar{\eta}]^{c}_{25} \xrightarrow{c \to 0\%}$: 16.5 dl/g

Protein content : 018% by wt./vol.

Zinc hyaluronate can be prepared as described above also from associates formed from other quaternary ammonium salts. Quaternary salts useful for this purpose are e.g.:

a) carbotetradecyloxymethyl-trimethylammonium chloride (see the Hungarian patent specification No. 188,537),

b) hexadecylpyridinium chloride,

c) cetylpyridinium chloride,

d) trimethylammonium chloride and the like.

Example 7

Preparation of cobalt hyaluronate

the process described in Example 6 is followed, except that the hyaluronic acid quaternary ammonium associate is dissolved in a solvent couple consisting of a 2% by wt./vol. cobalt(II) chloride.$6H_2O$ aqueous solution

and n-butanol.

Example 8

Preparation of an aqueous solution containing 0.50% by wt./vol. of zinc hyaluronate made isotonic by zinc chloride

About 50 ml of a zinc chloride solution of 0.110 mol/litre concentration are added to 0.50 g of sodium hyaluronate in a 100 ml volumetric flask and then allowed to swell overnight. Then, the sodium hyaluronate is dissolved by shaking and the flask is filled up to the mark with a zinc chloride solution of 0.110 mol/litre concentration.

The osmotic pressure of the solution obtained is 0.1491 mol/litre as expressed in equivalent sodium chloride concentration, the value of pH is 5.0. When necessary, the pH value is adjusted to 5.5 to 5.6 by adding 2.00 ml of a sodium acetate solution of 0.150 mol/litre concentration. After adjusting the pH value, the osmotic pressure of the solution is 0.1489 as expressed in equivalent sodium chloride concentration.

The zinc hyaluronate solution is prepared from the particularly pure sodium hyaluronate described in Example 3 with twice distilled water under aseptic conditions, then the solution is filtered through a membrane filter (0.45 μ pore size).

The solution obtained can be used in injectable compositions, too.

Example 9

Preparation of an aqueous solution containing 0.2% by wt./vol. of zinc hyaluronate made isotonic by zinc chloride

For a final volume of 100 ml, 0.20 g of sodium hyaluronate is weighed in and dissolved in a zinc chloride solution of 0.120 mol/litre concentration.

The dissolution and preparation of the zinc chloride solution of precisely 0.120 mol/litre concentration are carried out according to Example 1 (according to the sense by changing the amount of zinc chloride).

The osmotic pressure of the solution is 0.154 mol/litre as expressed in equivalent sodium chloride concentration; the pH shows a value of 5.3 to 5.4.

HA content	: 1.96 mg/ml
Viscosity	: 15.9 dl/g
Protein concent	: 0.015 mg/ml
Purity of the solution[x]	: $A_{660}^{1\,cm} = 0.015$

[x] Based on the absorbance measured at 660 nm in an 1 cm cuvet

The solution is prepared by using the sodium hyaluronate of the quality characterized in Example 2 and used first of all for the preparation of dermatologic and cosmetic compositions.

Example 10

Preparation of an aqueous solution containing 0.50 % by wt./vol. of zinc hyaluronate made isotonic by glucose

The solution of this Example contains sodium hyaluronate and the calculated equivalent amount of zinc chloride.

12.50 ml of a zinc chloride solution of 0.100 mol/litre concentration are added to 0.50 g of sodium hyaluronate weighed in a 100 ml volumetric flask. (An other concentration of zinc chloride may also be used but the amount of zinc chloride should be the same.) Sodium hyaluronate is allowed to swell for 12 hours in the solution of zinc chloride filled up to 50 ml with ion-free water, then dissolved by shaking. Thereafter, 24.50 ml of a glucose solution of 1.00 mol/litre concentration are added and filled up to the mark with ion-free water.

The osmotic pressure of the solution is 0.1495 mol/litre as expressed in equivalent sodium chloride concentration; the pH shows a value of 5.4. Total zinc concentration = 1.25×10^{-2} mol/litre.

The solution is prepared by using the sodium hyaluronate of the quality characterized in Example 2 and used first of all for the preparation of dermatologic and cosmetic compositions.

Example 11

Preparation of an aqueous solution containing 0.2 % by wt./vol. of zinc hyaluronate made isotonic by glucose

The solution of this example contains sodium hyaluronate and the calculated equivalent amount of zinc chloride.

5.0 ml of a zinc chloride solution of 0.100 mol/litre concentration are added to 0.20 g of sodium hyaluronate weighed in a 100 ml volumetric falsk, then the volume is completed to 50 ml with deionized water. After allowing to swell overnight, sodium hyaluronate is dissolved by shaking, 27.0 ml of a glucose solution of 1.00 mol/litre concentration are added and the flask filled up to the mark with ion-free water.

The osmotic pressure of the solution is 0.151 mol/litre as expressed in equivalent sodium chloride concentration; the pH shows a value of 5.6 to 5.7; Total zinc concentration = 5×10^{-3} mol/litre.

Example 12

Preparation of an aqueous solution containing 0.5 % by wt./vol. of zinc hyaluronate made isotonic by sorbitol

The zinc hyaluronate solution described hereinafter is prepared under aseptic conditions from sodium hyaluronate of particularly high purity described in Example 3 and distilled water. The solution contains zinc chloride in an equivalent amount calculated for sodium hyaluronate.

The process described in Example 10 is followed, except that, instead of the glucose solution, 23.50 ml of a sorbitol solution of 1.00 mol/litre concentration (182.19 g of D-sorbitol in 1 litre) are added to the zinc hyaluronate solution.

The solution thus prepared is filtered through a membrane filter (0.45 μ pore size). This solution can be used for any purpose including injectable compositions.

The osmotic pressure of the solution is 0.1520 mol/litre as expressed in equivalent sodium chloride concentration; the pH shows a value of 5.5; Total zinc concentration = 1.25×10^{-2} mol/litre.

Example 13

Preparation of an aqueous solution containing 0.2% by wt./vol. of zinc hyaluronate made isotonic by sorbitol

The solution described in this Example contains zinc chloride in an equivalent amount calculated for sodium hyaluronate.

The zinc hyaluronate solution described hereinafter is prepared under aseptic conditions from sodium hyaluronate of particularly high purity described in Example 3 with twice distilled water.

The process of Example 12 is followed, except that 0.2 g of sodium hyaluronate is dissolved, 5 ml of zinc chloride solution of 0.100 mol/litre concentration, then 26.50 ml of a sorbitol solution of 1 mol/litre concentration are added and finally, the solution is filled up to 100 ml. The solution thus prepared is filtered through a membrane filter (0.45 /u pore size). This solution can be used for any purpose including injectable compositions.

The osmotic pressure of the solution is 0.1501 mol/litre as expressed in equivalent sodium chloride concentration; the pH shows a value of 5.6;

Total zinc concentration = 5×10^{-3} mol/litre.

Hyaluronate content	: 2.03 mg/ml
Viscosity	: 16.1 dl/g
Protein content	: 0.016 mg/ml
Purity of the solution[x]	: $A_{660}^{1\ cm} = 0.010$

[x] Based on the absorbance measured at 660 nm in an 1 cm cuvet

Examples 14 to 26

In the following Examples the components of various compositions (pharmaceutical and cosmetic compositions) are given in relation to formulation types selected by us. The preparation of zinc hyaluronate solutions made isotonic are described in the preceding Examples. Here, "distilled water for injection purpose" means twice distilled water prepared under aseptic conditions.

I. Injectable solutions

Compositions of Examples 14 to 17 are used for intracutaneous administration whereas that of Example 18 serves for intraocular use. The active ingredient of the quality described in Example 3 is employed in these Examples.

Example 14

Zinc hyaluronate active ingredient	2.0 mg
Sorbitol	48.3 mg
Final volume of the aquous solution prepared with distilled water for injection purpose	1.0 ml

Example 15

Zinc hyaluronate active ingredient	5.0 ml
Sorbitol	42.8 mg
Final volume of the aqueous solution prepared with distilled water for injection purpose	1.0 ml

Example 16

Zinc hyaluronate active ingredient	2.0 mg
Propyl p-hydroxybenzoate	0.05 mg
Methyl p-hydroxybenzoate	0.5 mg
Glucose	48.6 mg
Final volume of the aqueous solution prepared with distilled water for injection purpose	1.0 ml

Example 17

Zinc hyaluronate active ingredient	5.0 mg
Propyl p-hydroxybenzoate	0.05 mg
Methyl p-hydroxybenzoate	0.5 mg
Glucose	44.1 mg
Final volume of the aqueous solution prepared with distilled water for injection purpose	1.8 ml

Example 18

Zinc hyaluronate active ingredient	10.0 mg
Potassium sorbate	1.0 mg
Sorbitol	41.0 mg
Final volume of the aqueous solution prepared with distilled water for injection purpose	1.0 ml

Compositions described in Examples 20 to 28 are mainly used for dermatologic and cosmetic purposes. The active ingredient of the quality described

in Example 2 is employed in these Examples.

II. Solutions for topical use

Example 19

Zinc hyaluronate active ingredient	5.0 mg
Potassium sorbate	1.0 mg
Sodium acetate	24.6 mg
Final volume of the aqueous solution prepared with distilled water	1.0 ml

Example 20

Zinc hyaluronate active ingredient	2.0 mg
Potassium sorbate	1.0 mg
Sorbitol	48.3 mg
Final volume of the aqueous solution prepared with distilled water	1.0 ml

III. Gels for topical use

Example 21

Zinc hyaluronate active ingredient		20.0 mg
Acrylic acid polymerisate		200 mg
Sodium hydroxide of 30% concentration		50 mg
Potassium sorbate		10 mg
Distilled water	up to	10.0 mg

Example 22

Zinc hyaluronate active ingredient		20.0 mg
Acrylic acid polymerisate		50 mg
Sodium hydroxide of 30% concentration		40 mg
Propylene glycol		1500 mg
Potassium sorbate		10 mg
Distilled water	up to	10.0 mg

IV. Creams and ointments for topical use

Example 23

Zinc hyaluronate active ingredient		50 mg
Potassium sorbate		10 mg
Soft white bee wax		125 mg
Sorbitan oleate		150 mg
Cetyl stearyl alcohol		840 mg
Glyceryl monostearate		1100 mg
Propylene glycol		4750 mg
Distilled water	up to	10 g

Example 24

Cobalt hyaluronate active ingredient		50 mg
Potassium sorbate		10 mg
Soft white bee wax		125 mg
Sorbitan oleate		150 mg
Cetyl stearyl alcohol		840 mg
Glyceryl monostearate		1100 mg

	Propylene glycol		4750 mg
	Distilled water	up to	10 g

Example 25

	Zinc hyaluronate active ingredient		50 mg
	2-Phenoxyethanol		100 mg
	Sodium lauryl sulfate		100 mg
	Cetyl palmitate		400 mg
	Stearin		400 mg
	Stearyl alcohol		450 mg
	Cetyl alcohol		450 mg
	White vaseline		500 mg
	Propylene glycol		550 mg
	Glycerol		600 mg
	Distilled water	up to	10.0 g

Example 26

	Cobalt hyaluronate active ingredient		50 mg
	2-Phenoxyethanol		100 mg
	Sodium lauryl sulfate		100 mg
	Cetyl palmitate		400 mg
	Stearin		400 mg
	Stearyl alcohol		450 mg
	Cetyl alcohol		450 mg
	White vaseline		500 mg
	Propylene glycol		550 mg
	Glycerol		600 mg
	Distilled water	up to	10 g

Example 27

Zinc hyaluronate active ingredient		50.0	mg
Microcrystalline wax		250	mg
Propylene glycol		500	mg
Sorbitol		400	mg
Wool wax (acetylated)		500	mg
White vaseline	up to	10	g

V. Compositions for the purification and cicatrizaton of purulent wounds and burns

Example 28

Zinc hyaluronate active ingredient		10	mg
Potassium sorbate		1.0	mg
Hydrophilic colloidal silicon dioxide		50	mg
Sorbitol	up to	1	g

Claims

1. Associates (complexes) of deprotonated hyaluronic acid with 3d metal ions of the 4th period of the periodic table.

2. An associate as claimed in claim 1, which comprises zinc ion as 3d metal ion.

3. An associate as claimed in claim 1, which comprises cobalt ion as 3d metal ion.

4. A pharmaceutical composition, which comprises as active ingredient an effective amount of an associate (complex) of deprotonated hyaluronic acid with 3d metal ions of the 4th period of the periodic table in admixture with carriers and/or additives commonly used in the pharmaceutical industry, optionally with (an) other therapeutically active agent(s).

5. A pharmaceutical composition as claimed in claim 4, which comprises the associate of deprotonated hyaluronic acid with zinc ion as active ingredient.

6. A cosmetic composition (having curative effect), which comprises as active ingredient an effective amount of an associate (complex) of deprotonated hyaluronic acid with 3d metal ions of the 4th period of the periodic table and optionally other additives.

7. A cosmetic composition, which comprises the associate of deprotonated hyaluronic acid with zinc ion as active ingredient or carrier.

8. A process for the preparation of novel associates (complexes) of deprotonated hyaluronic acid with 3d mteal ions of the 4th period of the periodic table, which comprises

a) adding an aqueous solution containing the equivalent amount of a salt, preferably the chloride of one of 3d metal ions of the 4th period of the periodic table to an aqeuous solution of sodium hyaluronate or to an other salt (alkaline or alkaline earth metal salt, optionally silver salt) of hyaluronate; or

b) dissolving an associate formed from hyaluronic acid with a quaternary ammonium salt in an aqueous suspension in a solvent couple containing the aqueous solution of a 3d metal ion of the 4th period of the periodic table and a solvent which is partially miscible with water, preferably n-butanol; then

- precipitating the associate (complex) obtained of deprotonated hyaluronic acid with the 3d metal ion of the 4th period of the periodic table by an alkanol or alkanone in a known manner, or
- separating the precipitate from the solution and then, if desired
- drying it under mild conditions.

9. A process as claimed in claim 8, which comprises using zinc ion as 3d metal ion.

10. A process as claimed in claim 8, which comprises using cobalt ion as 3d metal ion.

11. A process for the direct preparation of an aqueous composition containing zinc hyaluronate, which comprises adding an aqueous solution containing zinc chloride in an equivalent amount or in an amount exceeding the equivalent amount needed to reach the isotonic state.

12. A process for the preparation of a pharmaceutical composition, which comprises mixing or dissolving as active ingredient an associate of deprotonated hyaluronic acid with 3d metal ions of the 4th period of the periodic table, prepared by using the process according to claim 8, with commonly used carriers, diluents and optionally with an isotonizing agent or other additives and transforming them to a pharmaceutical composition.

13. A process for the preparation of a cosmetic composition, (having curative effect), which comprises mixing as active ingredient an associate of deprotonated hyaluronic acid with 3d metal ions of the periodic table, prepared by using the process according to claim 8, with commonly used carriers and diluents and optionally mixing or dissolving them together with an isotonizing agent and/or other additives and transforming them to a cosmetic composition.

14. A process as claimed in any of claims

12 or 13, which comprises using zinc associate as an associate formed with 3d metal ions of the 4th period of the periodic table.

15. A process as claimed in any of claims 12 or 13, which comprises using cobalt associate as an associate formed with 3d metal ions of the 4th period of the periodic table.

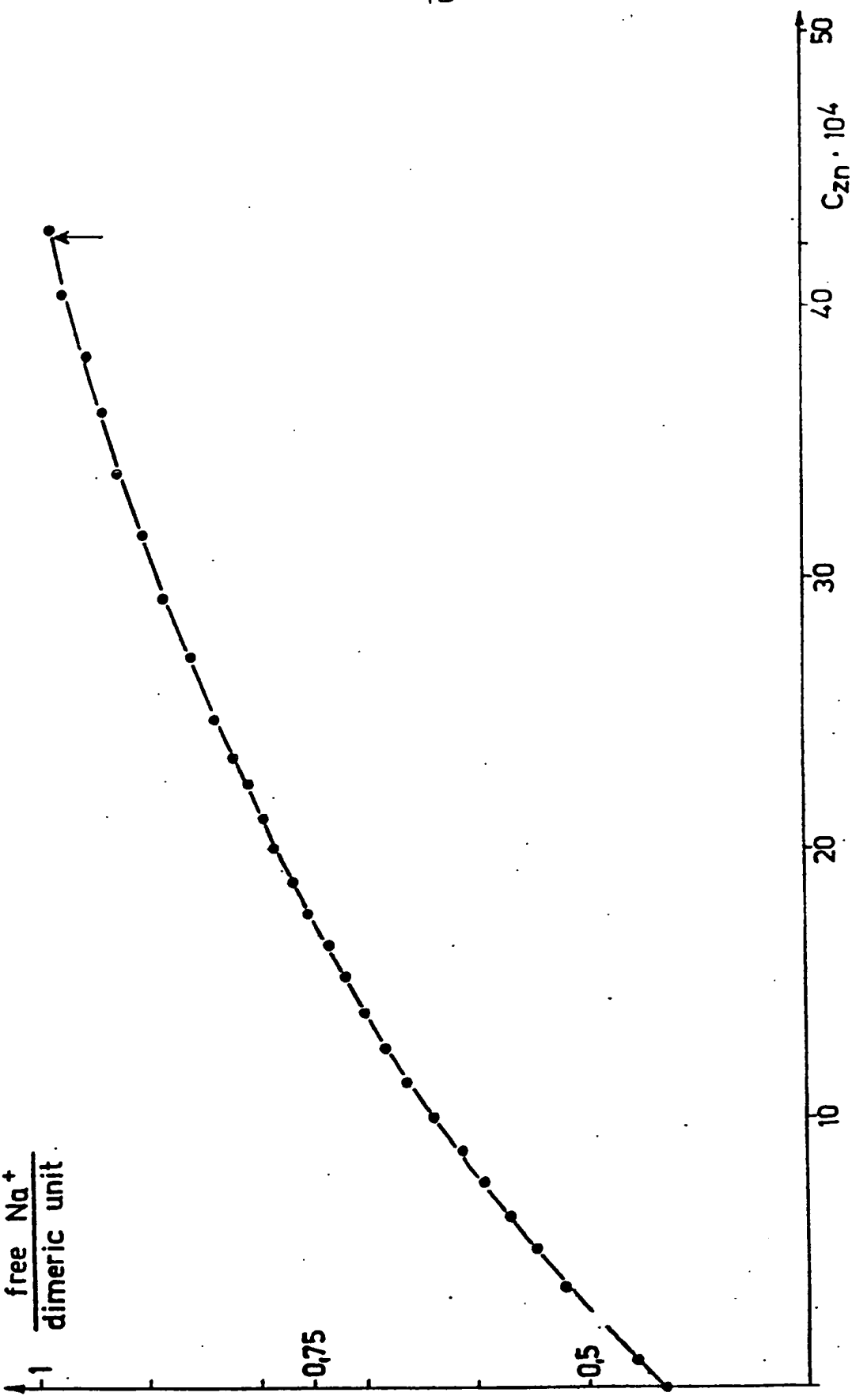
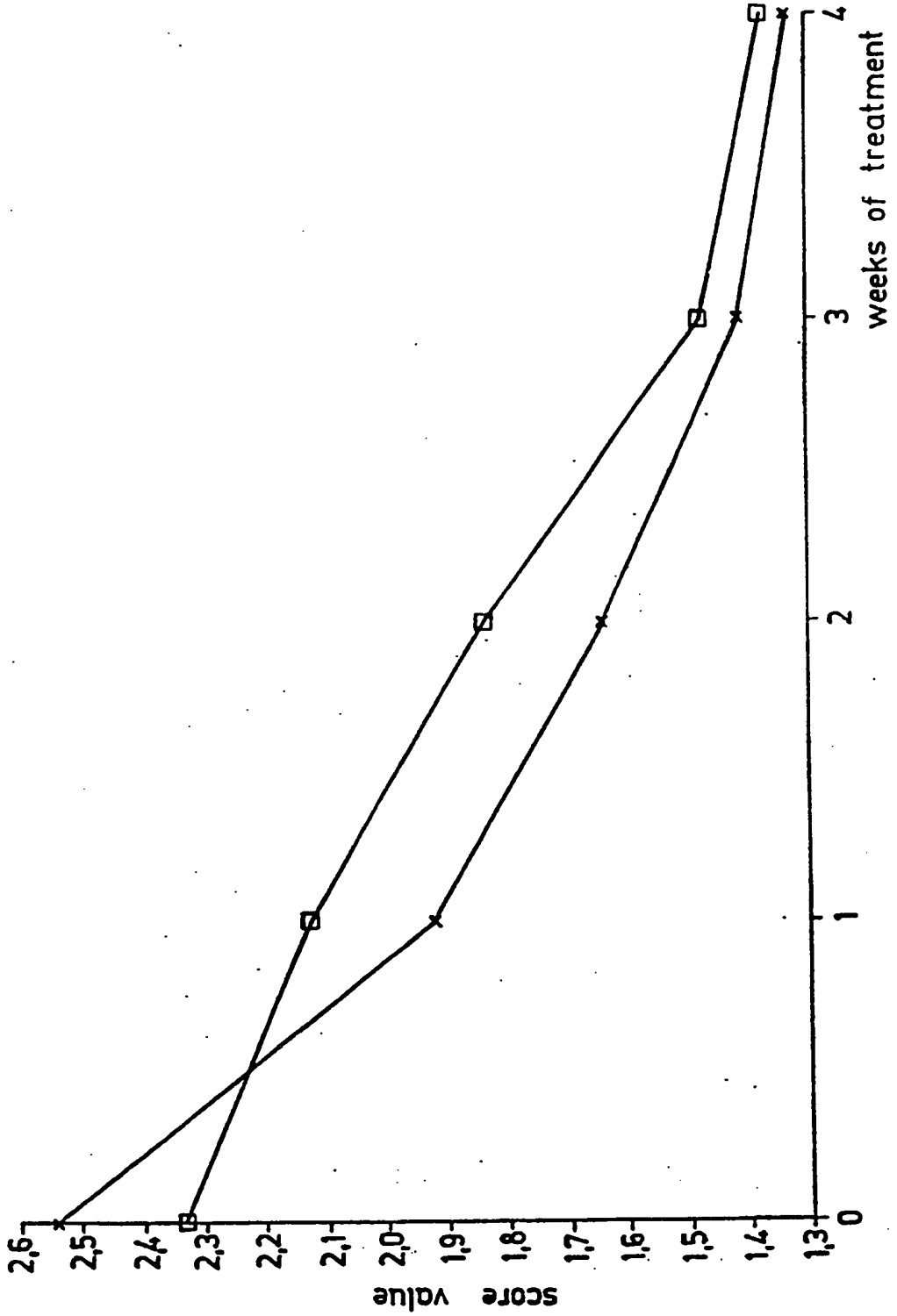


Fig. 1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0
1
2
3
4
weeks of treatment

Fig.2

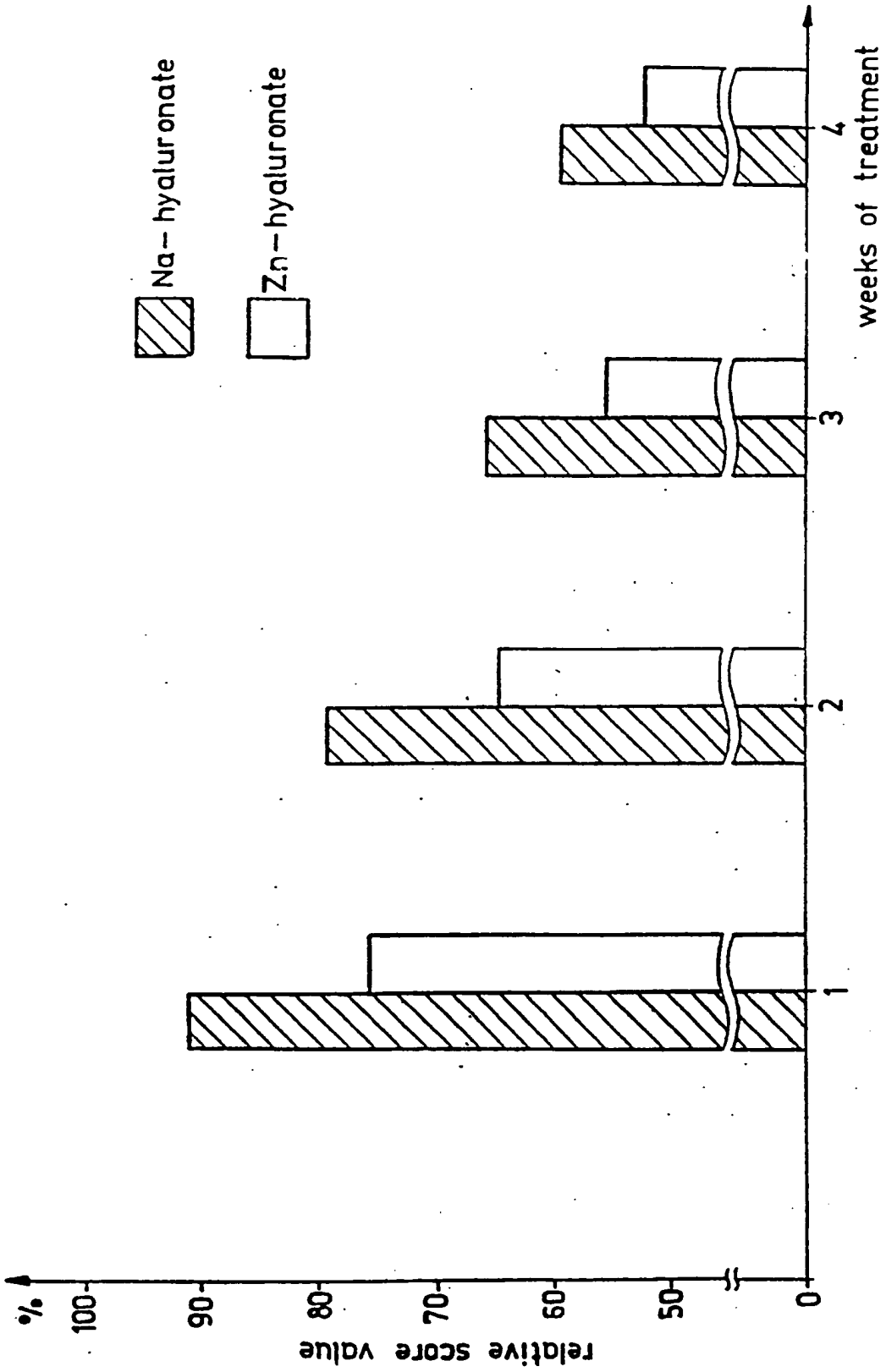
Na- hyaluronate
Zn- hyaluronate
weeks of treatment
1
2
3
4
relative score value
%
100
90
80
70
60
50
0

Fig. 3

INTERNATIONAL SEARCH RE T

International Application No PCT/HU 90/00013

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 08 B 37/08, A 61 K 31/715, A 61 K 33/30.

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
Int.Cl.[5]:	C 08 B 37/00; A 61 K 7/00, 31/00, 33/00.

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A1, 87/07 060 (ARTHROPHARM PTY. LIMITED) 22 September 1988 (22.09.88), see abstract; amended claims 1-3,5,9-13,18; page 23, line 11 - page 27, line 29.	(1,2,4-9, 12-14)
D,A	US, A, 4 746 504 (NIMROD et al.) 24 May 1988 (24.05.88), see column 3, line 33 - column 5, line 32. & WO,A1,87/05 517	(1,2,8,9)
D,A	The Biochemical Journal, London, Volume 193, 1981, R.F. Parrish and W.R. Fair: "Selective binding of zinc ions to heparin rather than to other glycosaminoglycans", pages 407-410, see abstract; page 409, table 1.	(1,2,8,9)

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* Special categories of cited documents: [14]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 April 1990 (25.04.90)	03 May 1990 (03.05.90)
International Searching Authority	**Signature of Authorized Officer**
AUSTRIAN PATENT OFFICE	

Anhang zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

Annex to the International Search Report on International Patent Application No. PCT/HU 90/00013

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned International search report. The Austrian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Annexe au rapport de recherche internationale relatif à la demande de brevet international n°.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche internationale visé ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsabilité de l'Office autrichien des brevets.

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO-A1- 8807060	22-09-88	AU-A1-15456/88 EP-A1- 356435 JP-A2- 1197100	10-10-88 07-03-90 08-08-89
US-A - 4746504	24-05-88	AU-A1-72068/87 DK-A - 5974/87 DK-A0- 5974/87 EP-A1- 259485 IL-A0- 81877 JP-T2-63502670 US-A - 4784991 WO-A1- 8705517 US-A - 4743876	09-10-87 13-11-87 13-11-87 16-03-88 20-10-87 06-10-88 15-11-88 24-09-87 10-05-88